A MOLECULAR MARKER BASED ON THE FRK2 (FRUCTOKINASE 2) GENE

FIELD OF THE INVENTION

The present invention relates generally to a method of breeding tomatoes having superior taste characteristics and to tomatoes having superior taste characteristics, and particularly to a molecular marker for a gene determining the fructose to glucose ratio in mature tomato fruit.

BACKGROUND OF THE INVENTION

Taste characteristics are a major determinant of fruit quality for both processing and fresh market tomatoes (see Stevens, M.A. 1986. Inheritance of tomato fruit quality components. Plant Breeding Reviews 4: 274-310). One of the major components of taste in tomatoes is soluble sugar content. The soluble sugar content of all known commercial cultivars of tomatoes (*Lycopersicon esculentum* Mill.) primarily includes the hexose sugars glucose and fructose in near-equimolar ratios (1:1 to 1:1.3) (see Davies J.N. and Hobson G.E. 1981. The constituents of tomato fruit- the influence of environment, nutrition and genotype, CRC Critical Review Food Science and Nutrition, 15:205-280; Davies J.N. and Kempton, R.J. 1975. Changes in the individual sugars of tomato fruit during ripening. J. Sci. Fd. Agric. 26: 1103-1110). In commercial *Lycopersicon esculentum* cultivars the disaccharide sucrose is also present but at concentrations rarely exceeding 0.5% on a fresh weight basis. Certain wild species of *Lycopersicon*, such as *L. hirsutum*, accumulate high concentrations of sucrose, which may reach 4% on a fresh weight basis (see Miron, D. and Schaffer, A.A. 1991. SPS, SS and invertase activities in developing fruit of *Lycopersicon esculentum* and the sucrose accumulating *L. hirsutum*. Plant Physiol. 95: 623-627). In the presence of high sucrose, these fruit accumulate low levels of the hexoses fructose and glucose, typically less than 1% each on a fresh weight basis (Davies J. N. On the Occurrence of Sucrose in *Lycopersicon* Fruit and its Nature, Nature, Vol. 266, 586-587, 1966). However, in these fruit the ratio of fructose to glucose is unusually high, more than 1.5:1.

Typically, plant breeders seek to improve the sweetness component of tomato flavor by increasing total soluble solids (TSS), measured by refractometric determination of a sample of juice and expressed as Brix. This measurement however does not differentiate between the component sugars. Fructose is significantly sweeter than both glucose and sucrose (see Biester, A.M., 1925. Carbohydrate studies: I. Relative sweetness of pure sugars. Amer. J. Physiology 73: 387-400). giving a tomato with a relatively high fructose content distinct advantages in terms of superior taste characteristics.

Tomatoes with high fructose to glucose ratios have been developed, using a method of selection described in applicant/assignee's US Patent application 5,817,913, the disclosure of which is incorporated herein by reference. In summary, this method consists of hybridizing a tomato plant of the *L. esculentum* species with a plant of the *L. hirsutum* species and in the subsequent progenies selection of mature fruit with fructose/glucose ratios of more than 1.8, together with fructose levels more than 1.3% on a fresh weight basis. The analysis of mature fruit sugars in the described method is via direct chemical analysis of the fruit sugars, for example by chromatographic separation of individual sugars.

Molecular markers have been used as a method of selection in plant and animal breeding, with obvious advantages (see Hillel J., Schaap T., Haberfeld A., Jeffreys A.J., Plotzky Y., Cahaner A. and Lavi U. 1990. Genomic selection: application of DNA fingerprints for efficient gene introgression. Genetics, 124:783-789; Tanksley, S.D., Ganal, M.W., Prince, J.P. et al. 1992. High density molecular linkage maps of the tomato and potato genomes. Genetics, 132: 1141-1160; Williamson V.M., Ho J.-Y., Wu F.F., Miller N. and Kaloshian I.. 1994. A PCR-based marker tightly linked to the nematode resistance gene, *Mi*, in tomato. Theor. Appl. Genet., 87:757-763; Chagu'e V., Mercier J.C., Gu'enard M., de Courcel A., and Vedel F. 1996. Identification and mapping on chromosome 9 of RAPD markers linked to *Sw-5* in tomato by bulked segregant analysis. Theor. Appl. Genet., 92:1045-1051). Several strategies to modulate sugar concentration and profile in ripe tomato fruit have been explored, including genetic approaches. However, precision breeding towards such directions involves assessment of reducing sugars carried out by HPLC (high pressure liquid chromatography) that is expensive and time consuming. DNA markers could potentially alleviate this problem, enabling the identification and selection of genetic material at the seedling stage, thus reducing significantly effort and time. During recent years, international efforts were invested aiming at the genome mapping of several plant species such as the tomato, potato and maize, using DNA markers (see Helentjaris T., Slocum M., Wright S., Schaefer A. and Neinhuis J. 1986. Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor. Appl. Genet., 72: 761-769; Tanksley et al., 1992). Apart from being an efficient tool for many breeding and genetic analyses (reviewed by Hillel J., Dunnington , E.A, and Siegel P.B. 1992. DNA markers in poultry breeding and genetic analysis. Poult. Sci. Rev., 4:169-186), DNA markers also provide initial sequence information and probes useful for cloning genes of interest. Recently, there were several successful reports of gene isolation and candidate gene identification in higher

plants by positional cloning (Tanksley, S.D., Ganal, M.W. and Martin, G.B. 1995. Chromosome landing: a paradigm for map-based cloning in plants with large genomes. Trends Genet., 11: 63-68; Folkertsma R.T., Spassova M.I., Prins M., Stevens M.R., Hille J. and Goldbach R.W. 1999. Construction of a bacterial artificial chromosome (BAC) library of *Lycopersicon esculentum* cv. Stevens and its application to physically map the *Sw-5* locus. Molecular Breeding 5:197-207)

Molecular linkage maps are largely composed of restriction fragment length polymorphism (RFLP) markers. RFLP analysis require a cloned probe, cleavage of genomic DNA with restriction endonucleases and time consuming DNA transfer, labeling and hybridization steps. More efficient polymorphism assays can be obtained from the polymerase chain reaction (PCR), that requires a substantially smaller amount of the analyzed DNA as compared to RFLP analysis (see Saiki R.K., Scharf S., Faloona F.A., Mullis K.B., Horn G.T., Erlich H.A. and Arnheim N. 1985. Enzymatic amplification of b-globin sequences and restriction site analysis for the diagnosis of sickle cell anemia. Science, 230:1350-1354). Several PCR-based marker identification techniques were developed and found useful in the detection of DNA sequences linked to genes of interest. These techniques include the random amplified polymorphic DNA (RAPD, see Williams J.G.K., Kublik A.R., Livak K.J., Rafalski J.A. and Tingey S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., 18: 6531-6535), microsatellite or simple sequence repeat analysis (SSR, see Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucl. Acids Res., 17: 6463-6471), inter SSR polymorphism using single primers of simple sequence repeats (see Gupata M., Chyi Y.-S., Romero-Severson J. and Owen J.L. 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor. Appl. Genet., 89:998-1006) and the technique of amplified restriction fragment polymorphism analysis (AFLP, see Zabeau M. and Vos P. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application 92402629.7 (Publication number: 0 534 858 A1)). The PCR techniques, mentioned above, can detect more subtle sequence polymorphisms than RFLP analysis and require only a small amount of DNA. RAPD and inter SSR analysis are low cost and easy to perform because no prior target DNA sequence information in polymorphic DNA regions is required for its implementation. These techniques, however, share the disadvantage of being able to usually identify only a single allele at any given locus and are therefore unable to discriminate

between homozygous and heterozygous genotypes. AFLP is more expensive to produce, can also usually detect only single allele at any given locus, but has the capacity to detect a much greater number of polymorphic loci in a single assay than other currently available PCR-based techniques. Microsatellites or SSR are also expensive to produce because they require allele specific primers, detect only a single polymorphic locus in a single assay but have the advantage of being able to identify more than one allele at any given locus and are therefore able to discriminate between homozygous and heterozygous genotypes.

PCR amplification analysis can be followed by restriction endonuclease cleavage of the amplification products in cases where length polymorphism can not be directly obtained in the PCR analysis of different genotypes. Such PCR analysis followed by endonuclease cleavage is often referred to as Cleaved Amplified Polymorphic Sequences (CAPS, see Konieczny A., and Ausubel F.M. 1993. A procedure for Mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J. 4:403-410; Jarvis P., Lister C., Szabo V. and Dean C. 1994. Integration of CAPS markers into the RFLP map generated using recombinant inbred lines of *Arabidopsis thaliana*. Plant Mol. Biol. 24:685-687), can detect more than one allele at any given locus and is therefore able to discriminate between homozygous and heterozygous genotypes.

In the case of selection for sugar content of mature fruit, a molecular marker has the advantage of allowing for selection at the young seedling stage, in contrast to selection only at the mature fruit stage. Furthermore, selection using a molecular marker eliminates the confounding effects of environmental influences on the plant phenotype which can limit the effectiveness of selection for a phenotypic trait such as mature fruit sugar content.

The enzyme fructokinase (EC 2.7.1.4) is able to phosphorylate fructose using a nucleoside triphosphate, such as ATP, as the substrate donating the phosphate moiety. As such, the enzyme may be able to modulate the ratio of fructose to glucose in plant tissue. At least two genes from *L. esculentum* that encode for divergent fructokinase enzymes, termed Frk1 and Frk2 have been cloned and sequenced (see Kanayama, Y., Dai, N., Granot, D., Petreikov, M., Schaffer, A and Bennett, A.B. 1997. Divergent fructokinase genes are differentially expressed in tomato. Plant Physiology 113: 1379-1384). The sequences for these two *L. esculentum* genes are described as Gene Bank Accessions U64817 and U64818, for Frk1 and Frk2, respectively.

It has been shown (Israel Application No. 121373, PCT Application No. PCT/IL98/00336, published application WO 99/04621) that wild species of *Lycopersicon* may

serve as sources of genetic variation for carbohydrate metabolism which may be utilized in the production of tomato plants producing fruit with modified carbohydrate metabolism and sugar content in the fruit.

In a previous patent application (Israel Application No. 121373, PCT Application No. PCT/IL98/00336, published application WO 99/04621) molecular markers associated with a locus in the tomato genome leading to an increase in fructose to glucose ratio in the mature tomato fruit were described. This locus was termed *Fgr* and is localized on tomato chromosome #4. (Levin, I., Gilboa, N., Yeselson, E., Shen, S. and Schaffer A.A. 1999. *Fgr*, a major locus that modulates fructose to glucose ratio in mature tomato fruit. Theor. Appl. Genet., in press).

SUMMARY OF THE INVENTION

The present invention seeks to provide a molecular marker for an additional gene which is operative to an increased fructose to glucose ratio in mature tomato fruit, as compared to the ratio generally present in standard tomato cultivars. In the present patent application we describe a molecular marker for an additional locus, located on tomato chromosome #6, in which the allele derived from a wild *Lycopersicon* species (*L. hirsutum*), modulates the fructose to glucose ratio in mature tomato fruit. The marker is for the gene Fructokinase 2 (*Frk2*), or for a gene linked to Frk2, whose wild species-derived allele increases the fructose to glucose ratio in mature tomato fruit and interacts with the previously characterized locus (*Fgr*), which is located on tomato chromosome number 4. The newly described marker, or the gene, can be used in conjunction with markers tagging the *Fgr* locus to produce tomato seeds, plants and/or fruit with the desirable characteristic of increased fructose to glucose ratio and to further increase this ratio.

There is thus provided in accordance with a preferred embodiment of the present invention an additional molecular marker for a gene or a gene determining fructose to glucose ratio in mature tomato fruit.

In accordance with a preferred embodiment of the present invention the marker includes an amplification product generated by primers called F2F and F2R primers, the F2F primer including a nucleotide sequence CGCCCGCTGAGTTGAATCTTGATCTT and the F2R primer including a nucleotide sequence CACAAGGACATGGCGGATTCATCATC. These primers are designed based on the nucleotide sequence of the gene encoding *Lycopersicon esculentum* fructokinase 2 (Genebank accession number U64818).

The marker that can be used to distinguish the *Frk2* gene originating from *Lycopersicon esculentum* as opposed to the *Frk2* gene originating from *Lycopersicon hirsutum* can be used to

increase fructose/glucose ratio because the *hirsutum* derived allele of the *Frk2* gene is associated with an increase in fructose to glucose ratio. It is reasonable that the marker, or a similar one, can distinguish the *Frk2* gene originating from *Lycopersicon esculentum* as opposed to the *Frk2* gene originating from other wild *Lycopersicon* species.

Further in accordance with a preferred embodiment of the present invention the marker includes at least part of or is at least part of a nucleotide sequence of the fructokinase 2 gene from *Lycopersicon hirsutum* as follows:

1 CATGGCAGTT AACGGTGCTT CTTCCTCTGG TTTGATCGTC AGTTTCGGTG AGATGTTGAT

61 CGATTTCGTT CCGACAGTCT CCGGCGTATC CCTTGCCGAG GCTCCCGGAT TTTTGAAAGC

121 TCCCGGCGGT GCACCGGCGA ACGTCGCTAT CGCGGTGACG AGGCTCGGAG GGAGGTCGGC

181 GTTCGTCGGG AAACTCGGCG ACGATGAGTT CGGTCACATG CTCGCCGGGA TTCTGAAAAC

241 GAACGGCGTA CAAGCCGATG GAATCAATTT TGACAAGGGC GCCAGGACGG CTTTGGCGTT

301 CGTGACTCTA CGCGCCGACG GAGAGCGTGA GTTTATGTTT TACAGAAATC CCAGTGCCGA

361 TATGTTGCTC ACGCCCGCTG AGTTGAATCT TGATCTTATT AGATCTGCTA AGGTGTTCCA

421 CTATGGATCA ATTAGTTTGA TCGTGGAGCC ATGTAGAGCA GCACATATGA AGGCAATGGA

481 AGTAGCTAAG GAGGCAGGGG CATTGCTCTC TTATGACCCT AACCTTCGTT TGCCGTTGTG

541 GCCTTCAGCA GAAGAAGCCA AGAAGCAAAT CAAGAGCATA TGGGACTCTG CTGATGTGAT

601 CAAGGTCAGC GATGTGGAGC TCGAATTCCT CACTGGAAGC AACAAGATTG ATGATGAATC

661 CGCCATGTCC TTGTGGCATC CTAACTTGAA GCTACTCTTG GTCACTCTTG GTGAAAAGGG

721 TTGCAATTAC TACACCAAGA AATTCCATGG AACCGTTGGA GGATTCCATG TGAAGACTGT

781 TGACACCACT GGAGCTGGTG ATTCTTTTGT TGGTGCCCTT CTAACCAAGA TTGTTGATGA

841 TCAAACCATT CTCGACGATG AAGCAAGGTT GAAGGAAGTA CTTAGGTTTT CATGTGCATG

901 TGGAGCCATC ACTACAACCA AGAAAGGAGC AATCCCAGCT TTGCCTACTG CATCTGAAGC

961 CCTCACTTTG CTCAAGGGAG GAGCATAGAA ACATCATGTT ATCTTTTTTC TTTTTTCCAT

1021 CTTCATATAT TTCCCCCCCT TTATGAGTTT TTTTTAACTT TGAAGCTAGT AGGAAGCCTT

Further in accordance with a preferred embodiment of the present invention the marker includes at least part of or is at least part of the amino acid sequence of the fructokinase 2 gene from *Lycopersicon hirsutum* as follows:

MAVNGASSSGLIVSFGEMLIDFVPTVSGVSLAEAPGFLKAPGGAPANVAIAVTRLGG

RSAFVGKLGDDEFGHMLAGILKTNGVQADGINFDKGARTALAFVTLRADGEREFMF
YRNPSADMLLTPAELNLDLIRSAKVFHYGSISLIVEPCRAAHMKAMEVAKEAGALLS
YDPNLRLPLWPSAEEAKKQIKSIWDSADVIKVSDVELEFLTGSNKIDDESAMSLWHP
NLKLLLVTLGEKGCNYYTKKFHGTVGGFHVKTVDTTGAGDSFVGALLTKIVDDQTI
LDDEARLKEVLRFSCACGAITTTKKGAIPALPTASEALTLLKGGA

Still further in accordance with a preferred embodiment of the present invention the amplification product generated by F2F and F2R is digested with the endonuclease *Eco*R I to generate a restriction fragment length polymorphism that distinguishes between the allele derived from *Lycopersicon hirsutum* and the allele derived from *Lycopersicon esculentum*.

There is also provided in accordance with a preferred embodiment of the present invention a method for breeding tomato plants that produce tomatoes having superior taste characteristics, including the steps of crossing at least one *Lycopersicon esculentum* plant with a *Lycopersicon* spp. to produce hybrid seeds, collecting the hybrid (F_1) seeds, growing plants from the F_1 seeds, pollinating the F_1 plants, collecting the hybrid seeds produced by the F_1 plants, growing plants from the seeds produced by the F_1 plants, measuring glucose and fructose content of ripe fruit produced from the plants grown from the seeds of the F_1 plants, providing a marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a *Lycopersicon* species, the marker being a marker for increased fructose/glucose ratio in tomato fruit, and using the marker to select a tomato plant with tomato fruit having desired characteristics including a fructose to glucose ratio greater than a ratio of standard *Lycopersicon esculentum*.

There is also provided in accordance with a preferred embodiment of the present invention a method for finding a gene, or a promoter region of a gene, that produces tomatoes having superior taste characteristics, including the steps of providing a marker that distinguishes a *Frk2* gene originating from *Lycopersicon esculentum* as opposed to a *Frk2* gene originating from a wild *Lycopersicon* species, the marker being a marker for increased fructose/glucose ratio in tomato fruit and using the marker to find the gene or the promoter region of said gene.

In accordance with a preferred embodiment of the present invention the method further includes cloning the gene.

Additionally in accordance with a preferred embodiment of the present invention the

method includes the step of propagating the plants with tomato fruits having the desired characteristics. Alternatively the plants may be propagated by vegetative propagation or by seed.

A tomato plant, tomato fruit and/or tomato seed may be produced in accordance with any of the methods of the present invention.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

Reference is now made to a method for selecting, in a breeding program, tomato plants with the genetic composition that determines that the mature fruit will have a fructose to glucose ratio higher than that found in standard tomato cultivars, on the average. The method of developing the plant material is as described in applicant/assignee's US Patent No. 5,817,913. Reference is now made to the following example which illustrates the invention.

Plant material description and analysis of sugar content in mature fruit

Parental lines of *Lycopersicon esculentum* differing significantly in their fructose to glucose ratio in the mature fruit were selected for this study, together with F_1, F_2 and F_3 populations generated by crossing the two parental lines. The high fructose to glucose ratio breeding line was derived from the introgression of the trait of high fructose to glucose ratio from the wild species *Lycopersicon hirsutum* (LA1777), as described in US Patent No. 5,817,913.

The following procedure was carried out for soluble sugar determination. Fruit portions of about 500 mg fresh weight were placed in 80% ethanol and soluble sugars were extracted from the tissue by heating to 70°C, as described in Miron and Schaffer (1991). Sugars were chromatographically separated by HPLC using a Bio-Rad Fast Carbohydrate column according to manufacturer's directions, as in Miron and Schaffer (1991). Sucrose glucose and fructose were identified by their retention times, refractometrically, and quantified in comparison to sugar standards.

Description of the PCR method, the PCR amplification marker generated by F2F and F2R primers and the analysis of the results

Genomic DNA was extracted from the 2 parental lines with divergent fructose to glucose ratio in the mature fruit and from individual plants of the F_1, F_2 and F_3 populations generated by crossing the two parental lines. The individual plants from the F_2 and F_3 population segregated for the trait of fructose to glucose ratio, the range being 1-2.5 in the F_2 population and 1.1-7.7 in the F_3 population. Individual plants from the F_2 and F_3 populations could therefore be easily

ranked for the trait of fructose to glucose ratio. The genomic DNA was extracted as in Fulton, T.M., Chunwongse, J. and Tanksley, S.D. 1995. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Molecular Biology Reporter 13: 207-209. In short, 50-100 mg of leaf tissue was ground in the presence of 2.5 parts DNA extraction buffer (0.35 M sorbitol, 0.1 M Tris-base, 5 mM EDTA, pH, 7.5); 2.5 parts nuclei lysis buffer (0.2 M Tris, 0.05 M EDTA, 2 M NaCl, 5% CTAB); 1 part 5% sarkosyl and 0.3 gm sodium bisulfite/100 ml. After incubation at 65 C for 120 min DNA was extracted with chloroform:isoamyl (24:1), precipitated with isopropanol, washed with 70% ethanol, dried and resuspended in ddH_2O.

F2F and F2R primers were synthesized (GibcoBRL, Inc., U.K.). These primers were designed based on the nucleotide sequence of the gene encoding *Lycopersicon esculentum* fructokinase 2 (Genebank accession number U64818). These primers were used in the presence of template DNA to screen by a polymerase amplification reaction for polymorphism between parental lines with divergent fructose to glucose ratios. The PCR products were digested with various restriction endonucleases and *Eco*R I was found to generate such restriction fragment length polymorphism.

Amplification reactions for the *Frk2* locus (25 µl final volume) contained 10 ng template DNA, 25 mM TAPS (pH 9.3 at 25°C), 50 mM KCl, 2mM MgCl2, 1 mM b-mercaptoethanol, 0.2 mM of each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 10 ng of each of two primers (F2F and F2R), and 1 unit of thermostable Taq DNA polymerase (SuperNova Taq Polymerase, MADI LTD., Israel). Reactions were carried out in an automated thermocycler (MJ Research Inc., Watertown, Massachusetts, USA). Initial incubation was at 94°C for 1.5 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1.5 min. Final polymerization at 72°C was carried out for 7 min after cycles were completed. The amplification products were visualized, after digestion with EcoRI (37°, 1 hour) according to manufacturer's recommendations, (New England Biolabs Inc., Beverly, MA, USA) by electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide. The genotype of each of the individual plants for the *Fgr* locus was determined as previously described in PCT published application WO 99/04621.

Genotype-phenotype relation

Analyses of variance were carried out using results obtained from the F_2 and F_3

population to determine the effect of association between each of the markers and the trait of fructose to glucose ratio and the percentage of fructose to glucose variation explained by these variation components. The DNA markers obtained were found highly and significantly associated with the trait of fructose to glucose ratio in both F_2 and F_3 populations (Tables 1,2,3 and 4). The association between both markers and the trait of fructose to glucose ratio was highly significant at a high log-of-differences (LOD) score explaining, together with a statistically significant interaction between them 48.5% and 61.9% of the total variation in fructose to glucose ratios observed in the F_2 and the F_3 populations, respectively (Table 2 and 4, respectively).

In conclusion, the results presented indicate that:

1. The DNA marker obtained by the amplification reactions using F2F and F2R primers is highly associated with an additional major gene encoding fructose to glucose ratios in the mature tomato fruits; and

2. The gene identified can directly or indirectly (through an interaction with the *Fgr* locus) modulate fructose to glucose ratios.

Table 1. Association between the fructokinase 2, *Fgr* and the trait of fructose to glucose ratio in F_2 population.

	Fructokinase 2		
Fgr	HH	HE	EE
HH	2.10 ± 0.09^{A}	1.74 ± 0.04^{B}	1.54 ± 0.06^{C}
HE	1.71 ± 0.05^{B}	1.49 ± 0.03^{C}	1.41 ± 0.02^{C}
EE	1.29 ± 0.04^{D}	1.24 ± 0.03^{D}	1.25 ± 0.02^{D}

It is noted that different letters represent statistically significant differences at the 0.05 level of significance. The letters E and H represent the derived genotypes of *esculentum* and *hirsutum*, and HE denotes the heterozygote thereof.

Table 2. Analysis of variance estimating the effects of fructokinase 2 locus, *Fgr* locus and the interaction between them on fructose to glucose ratio in the F_2 population.

Source of variation	Sum of squares	df	F ratio	Prob>F
Fructokinase 2 (*Frk2-II*)	1.49	2	20.84	5×10^{-9}
Fgr	6.09	2	84.84	4×10^{-28}
Frk2-II X *Fgr*	0.81	4	5.66	0.000232
Error	8.01	223		R^2=48.5

Table 3. Association between the fructokinase 2, *Fgr* and the trait of fructose to glucose ratio in F_3 population.

	Fructokinase 2		
Fgr	HH	HE	EE
HH	3.81 ± 0.15^{A}	2.26 ± 0.16^{C}	2.01 ± 0.06^{C}
HE	2.88 ± 0.18^{B}	1.76 ± 0.14^{D}	1.71 ± 0.02^{D}
EE	1.68 ± 0.17^{D}	1.30 ± 0.15^{E}	1.37 ± 0.02^{E}

Table 4. Analysis of variance estimating the effects of fructokinase 2 locus, *Fgr* locus and the interaction between them on fructose to glucose ratio in the F_3 population.

Source of variation	Sum of squares	df	F ratio	Prob>F
Fructokinase 2 (*Frk2-II*)	24.11	2	47.94	7×10^{-17}
Fgr	28.88	2	57.44	4×10^{-19}
Frk2-II X *Fgr*	7.88	4	7.83	0.000009
Error	38.22	152		R^2=61.9

Sequence of *Lycopersicon hirsutum* fructokinase II gene

Total RNA was extracted from young fruits (2 g fresh weight) of an individual plant homozygous for the fructokinase II allele derived from *Lycopersicon hirsutum* (*Frk2-II* ^{HH}). The RNA extraction was carried out using the TRIzol reagent system (GibcoBRL Life Technologies, Gaithersburg, MD, USA). The total RNA was used as template for first strand cDNA synthesis using the Superscript preamplification system (GibcoBRL Life Technologies, U.K.). The cDNA prepared was used as template in a PCR reaction to amplify four overlapping fragments of the

gene encoding fructokinase II (*Frk2* ^{HH}). The DNA fragments were excised from an agarose gel and purified using the GENECLEAN II kit (BIO 101 Inc., La Jolla CA, USA). The PCR fragments were then cloned into an pGEM-T Easy vector using the pGEM-T and pGEM-T Easy Vector Systems according to the manufacturer recommendations (Promega corporation, Madison, WI, USA). Four independent clones of each of the four amplified fragments were sequenced, based on both the T7 and SP6 complementary primers, using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The nucleotide sequence of the fructokinase II gene derived from *Lycopersicon hirsutum* (*Frk2*^{HH}) is as follows:

1 CATGGCAGTT AACGGTGCTT CTTCCTCTGG TTTGATCGTC AGTTTCGGTG AGATGTTGAT

61 CGATTTCGTT CCGACAGTCT CCGGCGTATC CCTTGCCGAG GCTCCCGGAT TTTTGAAAGC

121 TCCCGGCGGT GCACCGGCGA ACGTCGCTAT CGCGGTGACG AGGCTCGGAG GGAGGTCGGC

181 GTTCGTCGGG AAACTCGGCG ACGATGAGTT CGGTCACATG CTCGCCGGGA TTCTGAAAAC

24GAACGGCGTA CAAGCCGATG GAATCAATTT TGACAAGGGC GCCAGGACGG CTTTGGCGTT

301 CGTGACTCTA CGCGCCGACG GAGAGCGTGA GTTTATGTTT TACAGAAATC CCAGTGCCGA

361 TATGTTGCTC ACGCCCGCTG AGTTGAATCT TGATCTTATT AGATCTGCTA AGGTGTTCCA

421 CTATGGATCA ATTAGTTTGA TCGTGGAGCC ATGTAGAGCA GCACATATGA AGGCAATGGA

481 AGTAGCTAAG GAGGCAGGGG CATTGCTCTC TTATGACCCT AACCTTCGTT TGCCGTTGTG

541 GCCTTCAGCA GAAGAAGCCA AGAAGCAAAT CAAGAGCATA TGGGACTCTG CTGATGTGAT

601 CAAGGTCAGC GATGTGGAGC TCGAATTCCT CACTGGAAGC AACAAGATTG ATGATGAATC

661 CGCCATGTCC TTGTGGCATC CTAACTTGAA GCTACTCTTG GTCACTCTTG GTGAAAAGGG

721 TTGCAATTAC TACACCAAGA AATTCCATGG AACCGTTGGA GGATTCCATG TGAAGACTGT

781 TGACACCACT GGAGCTGGTG ATTCTTTTGT TGGTGCCCTT CTAACCAAGA TTGTTGATGA

841 TCAAACCATT CTCGACGATG AAGCAAGGTT GAAGGAAGTA CTTAGGTTTT CATGTGCATG

901 TGGAGCCATC ACTACAACCA AGAAAGGAGC AATCCCAGCT TTGCCTACTG CATCTGAAGC

961 CCTCACTTTG CTCAAGGGAG GAGCATAGAA ACATCATGTT ATCTTTTTTC TTTTTTCCAT

1021 CTTCATATAT TTCCCCCCCT TTATGAGTTT TTTTTAACTT TGAAGCTAGT AGGAAGCCTT

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described hereinabove. Rather the scope of the present invention includes both combinations and subcombinations of the features described hereinabove as well as modifications and variations thereof which would occur to a person of skill in the art upon reading the foregoing description and which are not in the prior art.